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Cancer Chemotherapy and Biological Response Modifiers Annual 17

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CHAPTER 4

Taxanes

Dan Sackett and Tito Fojo

1. Introduction

For oncologists discouraged with the progress of chemotherapy in the treatment of solid tumors, the taxanes, especially paclitaxel, have provided both hope and insight. Hope, knowing that agents can be found that are active against a wide range of solid tumors; and insight into both drug–target interaction and cellular response at a molecular level.

In the present chapter we will describe recent progress in taxane development. Clinically, the broad activity of this class of agents has been confirmed and their use in combination regimens is increasing. In ovarian cancer, the rapid incorporation of paclitaxel in up-front regimens was a recognition of its activity in this disease, and the need to diversify chemotherapeutic regimens to incorporate drugs from more than one class. It is the latter that makes taxanes so appealing: an active agent that does not belong to the anthracycline, alkylating or antimetabolite class of drugs, which are the cornerstones of so many regimens.

While the clinical development of taxanes has been most exciting to oncologist, increased understanding of this class of drugs has also been of interest because of the broad range of activity and the unique mechanism of action. The observations that in contrast to results with other chemotherapeutic agents and ionizing radiation, p53 mutations do not predict/preclude response to paclitaxel, has provided both a rationale for the activity of this class of compounds in a broad range of cancers, and also stimulated studies to understand alternate cytotoxic pathways.

The length of this chapter reflects the intense interest in these compounds at the present time. As the hope and promise of early trials become a reality, it is clear that these agents are now part of the oncologist's armamentarium.

2. Basic research

2.1. *Interaction with microtubules*

The nature of the interaction of paclitaxel with microtubules continues to be clarified. In tubulin, the location of the binding site peptides has been further defined, with residues from at least two sites in β-tubulin implicated in paclitaxel binding: residues from the first 30 were identified using a photoaffinity probe located at the 3′ moiety of the A ring C-13 side chain [1]; while residues 217–231 were labelled when the position of the photoaffinity probe was moved to the B ring C-2 side chain of paclitaxel [2].

In microtubules, the location of bound drug has been clarified using electron crystallogra-

phy and cryomicroscopy. Electron crystallography of tubulin–zinc sheets has revealed that bound paclitaxel is located on the outer surface of the microtubule, near the inter-protofilament contact but not near the intra-dimer or inter-dimer subunit contact sites [3], supporting models of drug action that invoke changes of conformation of individual tubulin dimers in the microtubule that alter lateral interactions, but do not rule out bridging interactions across the protofilament interface. Electron cryomicroscopy of paclitaxel-treated microtubules reveals an alteration of the protofilament number and a slight increase in the subunit spacing along the protofilament, an effect that is less pronounced if paclitaxel is added to preformed microtubules instead of being present during polymerization. These results might indicate that paclitaxel stabilizes tubulin dimers in the microtubule core in the GTP conformation rather than the usual (and slightly shorter) GDP conformation [4].

But binding must alter dynamics for a biological effect to occur. Microtubules are dynamic structures, growing and shortening frequently and paclitaxel binding stabilizes microtubules by suppressing these dynamic changes. Monitoring the dynamics of individual microtubule revealed that substoichiometric bound paclitaxel (0.001–0.01 paclitaxel per microtubule tubulin dimer) significantly decreased the rate and extent of shortening events. These concentrations of paclitaxel induced small increases in total polymer, and had little effect on growing rates. These results favor a paclitaxel-induced conformational change in the dimer to which it is bound which renders that dimer poorly able to dissociate [5]. Such a conformation would be similar in this regard to the GTP-state, with paclitaxel-binding occurring predominantly in the GDP-tubulin core of the microtubule, rather than at the ends. Binding to the exposed ends requires much higher paclitaxel concentrations, which may explain why higher paclitaxel is required to increase the total microtubule polymer in cells than is required to inhibit microtubule function [5,6].

2.2. *Chemical structure, taxane biosynthesis, and analog development*

The complex diterpene structure of paclitaxel and docetaxel is now familiar to many but is still conveniently discussed if represented consistently. With the ester side chain of C-13 at the left, the four rings of the taxane core proceed A to D, ending with the four-membered oxetane ring D on the right. With this orientation, and with C-1 down, the upper region of the structure is often named the 'North' and the lower, C-1 region the 'South.'

The three-dimensional structure of paclitaxel has been solved from the crystal. The taxane ring structure is very similar to that previously found for docetaxel, but the C-13 side chain conformation is different, being extended in paclitaxel, but more folded in docetaxel. Several groups on the molecule identified as important in structure–activity studies participate in hydrogen bonding in the crystal. These include the C-2′ hydroxyl and the C-2 benzoate groups [7].

Taxane biosynthesis is increasingly being clarified. The first step in natural production of paclitaxel and other taxanes has been shown to be the cyclization of geranylgeranyl diphosphate, and the enzyme responsible, taxadiene synthase, has been purified and characterized. Beginning with stems from Pacific yew saplings, the enzyme was purified about 600-fold and characterized as a monomer of M_r 79 000, with a pH optimum of 8.5 that requires Mg^{2+} for activity. It catalyzes the condensation of geranylgeranyl diphosphate to taxa-4(5),11(12)-diene. When this product was ^{3}H-labeled and incubated with stem sections,

a series of functionalized taxanes were produced, including baccatin III, cephalomanine, paclitaxel, and their 10-deacetyl derivatives. This work demonstrates that the first committed, and possibly rate-determining, step in the biosynthesis of paclitaxel is cyclization of geranylgeranyl diphosphate and opens the way to biological control of production of desirable taxanes [8,9].

In addition, new natural taxane diterpenoids with different skeletons from paclitaxel have been isolated from Japanese yew trees. These compounds, taxuspines A–D, consist of a 6/8/6 membered ring system and lack the A ring C-13 side chain as well as the four membered oxetane ring D of paclitaxel, both features important to the activity of paclitaxel. All are less cytotoxic than paclitaxel, although taxuspine D stabilizes microtubules against Ca^{+2}-induced depolymerization. Taxuspines A–C did not show such activity but did increase cellular accumulation of vincristine in multidrug-resistant human ovarian cancer cells [10,11].

Analog development has focused on the oxetane ring D region, the B ring C2 side chain in the 'South', the C13 side chain, and position C10 in the 'North'. The importance of the oxetane ring D region to paclitaxel action was reinforced by structure–activity studies of a series of D-ring C-4 side chain derivatives. Substitution of a cyclopropyl ester at this position increased potency in a tubulin polymerization assay and in vitro cytotoxicity against HCT-116 tumor cells by about 3-fold compared to paclitaxel. An isosteric aziridine ring in the same position caused a 10-fold loss of activity in both assays, indicating that apparently minor structural changes at this position can significantly alter potency [12]. In addition, analysis of a series of paclitaxel analogs with modifications in the B-ring C-2 side chain in the 'South' produced an analog with substantially increased activity. Substitution of a m-azidobenzoyl group for the benzoyl group present in paclitaxel increased activity in assays of tubulin polymer stability, as well as induction of polymerization at low temperature. Activity in promoting polymerization was greater than docetaxel which was greater than paclitaxel, and was partly due to increased nucleation. Polymer stabilization and hypernucleation of polymerization are separate features of taxane action, since other analogs that stabilized polymers did not show hypernucleation activity [13]. Finally, a series of very active paclitaxel analogs have been prepared in which changes are made in the C-13 side chain as well as in the 'North', at position C-10. The C-13 side chain 3′-phenyl was replaced with a 3′-methylpropyl or 3′-methylpropenyl group and coupled with several C-10 substituents. Most of the analogs showed greater activity than paclitaxel or docetaxel, with subnanomolar cytotoxicity against several cancer cell lines. The nature of the C-10 moiety did not strongly alter cytotoxicity but did greatly alter potency against MDR cell lines by as much as 100-fold compared to paclitaxel or docetaxel [14].

The water insolubility of paclitaxel and docetaxel has been a continuing concern. With other drugs, water-insolubility has been resolved by the production of phosphate water-soluble prodrugs. Consequently, a series of paclitaxel phosphate prodrugs have been synthesized and their properties before and after metabolic activation by alkaline phosphatase reported. The best results were obtained by derivatizing the C-2′ position of the C-13 side chain with a phosphonoxyphenylpropionate ester group. This compound was inactive in an in vitro microtubule polymerization assay, but recovered up to 100% of the activity of the same concentration of paclitaxel after activation with exogenous alkaline phosphatase. The prodrug was about 10-fold less active in a cytotoxicity assay using the human colon adenocarcinoma

cell line HCT-116, but toxicity was substantially restored by phosphatase activation. These findings show that water soluble paclitaxel prodrugs can yield normal paclitaxel activity following metabolic activation [15].

Beyond taxanes, two new compounds with paclitaxel-like activity but with structures unrelated to the taxanes have been described. Discodermolide is a marine, cytotoxic lactone-bearing, polyhydroxylated alkatetraene. It causes mitotic arrest and induces extensive microtubule bundling at much lower concentrations than paclitaxel. In in-vitro microtubule assembly assays it is also more potent than paclitaxel, and can induce near total polymerization on ice. It is likely a hypernucleating as well as a microtubule stabilizing compound [16]. The epothilones A and B are bacterial products with 16-membered macrolide skeletons. These agents induce mitotic arrest and microtubule bundling in fibroblasts, promote tubulin polymerization and stabilize microtubule polymers against cold and calcium. Their activity is similar to paclitaxel in all assays, and they are competitive inhibitors of paclitaxel binding to microtubule. Their activity is influenced less by P-glycoprotein, suggesting they are probably poor substrates [17].

2.3. *Cell type and cell cycle specificity*

Previous studies had demonstrated that paclitaxel was less cytotoxic to contact inhibited normal cells than to neoplastic cell lines in assays of growth inhibition and colony formation, suggesting transformed cells were more sensitive [18]. A similar increase in sensitivity to paclitaxel following transformation was reported in IMR90 cells transformed with SV40. A temperature-sensitive SV40 mutant conferred paclitaxel sensitivity only at the permissive temperature, and sensitivity corresponded to the presence of T-antigen [19].

In malignant cells, continuous exposure to toxic concentrations of paclitaxel results in G2/M accumulation but pulse exposure may be cytotoxic without mitotic block. Furthermore, the effects on synchronized HeLa cells of 1 h exposure to paclitaxel or docetaxel reveals differences in the drugs. Continuous exposure to either drug leads to prometaphase arrest, but a 1-h exposure does not, with sensitivity depending on when during the cell cycle drug exposure occurs. Using a clonogenic assay, drug effects at various points during the cell cycle of initially G1 synchronized cells were examined for 1 h exposure to drug concentrations that were IC_{50} in asynchronous culture assay (10 nM docetaxel or 225 nM paclitaxel). Toxicity of docetaxel was maximal and absolute during S-phase. Cells so treated progress through aberrant mitosis, became multinucleated and rounded, and died. In contrast, paclitaxel toxicity was minimal in early S and maximal in early M. Cell cycle differences in uptake were not observed, and cells accumulated docetaxel to about the same 400-fold previously found with paclitaxel, suggesting that different taxanes may target different cytotoxic mechanisms [20].

2.4. *Apoptosis*

The role of apoptosis in paclitaxel action has been addressed in a number of studies. While the initial action of paclitaxel and docetaxel is clearly stabilization of microtubules, the pathway between this and cell death is not clear. A number of studies make clear that paclitaxel or docetaxel exposure can lead to activation of apoptotic pathways, and such activation may represent a significant part, though not all, of the cytotoxic effect produced by these agents. The molecular details of apoptotic activation subsequent to microtubule stabilization are not clear, but appear to involve phosphoregu-

lation pathways. The role of p53 in paclitaxel cytotoxicity has been investigated, and it appears that mutation of p53 alone does not lead to drug resistance, as seen with other agents, most likely because p53 independent pathways exist. Paclitaxel-activated apoptosis may involve a pathway distinct from DNA-damaging agents, and investigation of the latter are actively ongoing with some studies suggesting this may involve Raf-1 and bcl-2.

Induction of apoptosis by paclitaxel has been demonstrated in many systems. These studies emphasize the similarity of apoptosis following paclitaxel to that following other agents, and underscore the role of tubulin in this process. Studies in human T-cell leukemia CCRF-CEM cells, with paclitaxel, methotrexate, or VP-16 found that in apoptotic cells both cytoplasmic protrusions and invaginated nuclei stained strongly for microtubule bands or bundles. In contrast, cells that accumulated in mitosis showed multiple asters or disordered mitosis but did not show apoptotic morphology. The fraction of cells with apoptotic morphology at the time of DNA fragmentation (about 40%), and tubulin changes were similar following treatment with paclitaxel or methotrexate or VP-16, suggesting that once triggered, the apoptotic pathway is similar and involves reorganization of tubulin [21]. Similar observations have been made in vivo by quantitating mitotic arrest and apoptosis histologically following paclitaxel treatment of mice bearing syngeneic tumors. Varying degrees of mitotic block were observed, but this did not correlate with growth delay, while apoptosis was not found in all cells, but did correlate with growth delay. Furthermore, basal (pretreatment) levels of apoptosis correlated with paclitaxel-induced apoptosis and with growth delay, suggesting that a propensity to apoptosis leads to paclitaxel sensitivity. Thus, sensitivity to paclitaxel inhibition of tumor growth was predicted by the induction of apoptosis, which in turn was correlated with the intrinsic rate of apoptosis in untreated cells [22].

In contrast, in mice bearing murine mammary carcinoma MCA-4 and ovarian carcinoma OCA-1 tumor, mitotic block was evident after 1 h after paclitaxel treatment and peaked after 9 h, with apoptosis beginning at 9 h, peaking at 18–24 h (about 20% of cells), and declining to baseline after 3–4 days. A second dose of paclitaxel at day 3 inhibited tumor growth equal to or better than the first dose, but caused little mitotic block or apoptosis, suggesting that while paclitaxel-arrested tumor cells may die by apoptosis, there may be other paclitaxel antitumor activities as well [23].

Studies in human myeloid leukemia cell lines are consistent with the observation of paclitaxel induced cell death independent of apoptosis. Treatment of HL-60, U937 and K562 cells with paclitaxel induces G2/M block in the three cell lines. However, DNA fragmentation, hypodiploid DNA content, and apoptotic morphological changes are not observed in K562 cells. Apoptosis is also not observed following exposure of K562 cells to other agents that induce apoptosis in HL-60 cells. These data support the thesis that paclitaxel induces apoptosis in susceptible cells. However, since paclitaxel causes mitotic arrest and death of K562 cells without apoptosis, it also indicates that paclitaxel can cause cell death via other pathway(s) [24].

When/where apoptosis occurs, the pathways and molecules involved remain to be elucidated. Several studies have implicated phosphorylation pathways in paclitaxel-initiated apoptosis, both by direct demonstration of modulation of phosphorylation and by the effects of agents known to alter phosphorylation. In the human ovarian tumor cell line OV2008, the protein tyrosine kinase inhibitor, genistein, inhibited paclitaxel-induced DNA fragmenta-

tion, reducing DNA fragmentation 10-fold and increasing the paclitaxel IC_{50} about 3-fold. Although genistein did not prevent paclitaxel-induced G2/M accumulation, it did prevent the paclitaxel-induced decrease in bcl-2, suggesting protein phosphorylation is not required for paclitaxel mitotic block but is necessary for induction of apoptosis by paclitaxel [25].

Similar results were obtained in human pre-B leukemia 697 cells. Co-treatment with either genistein or phorbol dibutyrate reduced paclitaxel-induced DNA fragmentation and apoptosis, but staurosporine, a protein kinase inhibitor, increased it. And as in OV2008 cells, none of these agents altered paclitaxel-induced mitotic arrest [26].

The role of p53 remains unclear. Induction following paclitaxel can occur, although the extent and significance are uncertain. An increase in transcriptionally active p53 was found in a gel mobility shift assay using nuclear extracts from 3T3 mouse fibroblasts treated for 24 h with varying concentrations of paclitaxel or vincristine or nocodazole. All three agents increased p53 DNA binding at concentrations that had little effect on cell cycle distribution. Higher concentrations that caused G2/M accumulation induced a maximal p53 increase. Treatment with vincristine and nocodazole led to induction of $p21^{WAF1}$ at the same concentrations that increased p53, but paclitaxel did not [27].

Similarly, a small induction of p53 was also observed by immunoblot in MCF-7 cells after exposure to low paclitaxel concentrations for 24 h. Increased levels of $p21^{WAF1}$ were also found. Paclitaxel exposure also increased levels of $p21^{WAF1}$ in p53-null PC3M human prostate cells, although to a lesser degree than was found with wild type p53. Changes in gel mobility indicated that paclitaxel exposure had also led to activation of Raf-1 and MAP kinase, Depletion of Raf-1 with geldanamycin prevented paclitaxel induction of p53 or $p21^{WAF1}$ or activation of MAP kinase, suggesting that paclitaxel induces $p21^{WAF1}$ [28].

While these studies demonstrate induction of p53 following paclitaxel exposure, it does not appear that a functional p53 is indispensable. For example, normal human fibroblasts depleted of p53 by SV40 T antigen or HPV-16E6, and mouse embryo fibroblasts (MEF) from p53-null mice were shown to be about 10-fold more sensitive to paclitaxel. Decreased p53 led to increased paclitaxel-induced G2/M accumulation, micronuclei formation, and DNA fragmentation; while cells with active p53 progressed through mitosis and transiently accumulated in G1 with increased levels of p53 and $p21^{WAF1}$. These results indicate that p53-mutant or p53-null cells exposed to paclitaxel are more sensitive to paclitaxel, are better blocked in G2/M, and undergo p53-independent apoptosis [19].

Slightly different results of p53 modulation were reported in a separate study with wild type and p53-null MEF. Following paclitaxel exposure, cells either arrested in prophase and underwent DNA fragmentation within 30 min, or escaped and arrested in G1 with multiple micronuclei, where they underwent DNA fragmentation only after several days. Both wt p53 and p53-null MEF cells that arrested in prophase rapidly showed DNA fragmentation. But p53-null cells that escaped mitotic block and became multinucleate continued to cycle through several rounds of mitosis without cytokinesis and without DNA fragmentation; while multinucleate wt-p53 cells remained arrested in G1 and exhibited DNA fragmentation at days 3–6. These results indicate that paclitaxel can induce two apoptotic pathways: a p53-independent, rapid onset pathway following mitotic block, and a p53-dependent pathway that is slow and follows G1-block [29].

Further support of the observation that paclitaxel can induce apoptosis independent of p53 has been obtained in clinical studies in patients with non-small cell lung carcinoma (NSCLC) treated with paclitaxel and radiotherapy. Mutant p53 was found in 40% of 30 stage-IIIA or -IIIB NSCLC patients but response rates (complete plus partial) in patients with tumors bearing mutant p53 (75% response) or wild type p53 (83% response) were not significantly different ($P = 0.70$). These results indicate that p53 status is not predictive of response to paclitaxel, indicating that paclitaxel can lead to cell death by pathway(s) that bypass p53, and suggesting that paclitaxel may be active against tumors with p53 mutations [30].

While the studies examining the relation of p53 and paclitaxel treatment/response may appear contradictory, they can be reconciled by the existence of both p53-dependent and p53-independent cytotoxic pathways. And because the latter may be more important and could potentially function in all cells, the nature of the p53-independent apoptosis pathway(s) has been pursued in several studies. In these investigations, bcl-2 and related proteins that are known to modulate apoptosis have emerged as potentially important. For example, treatment of pre-B lymphoid cells with either okadaic acid or paclitaxel has been shown to result in phosphorylation of bcl-2 and initiation of apoptosis. Cells that did not phosphorylate bcl-2 did not undergo apoptosis, suggesting that the anti-apoptotic activity of bcl-2 is lost by phosphorylation [31]. While in this study overexpression of bcl-2 did not prevent paclitaxel or okadaic acid induced apoptosis, in a separate study, bcl-2 overexpression was found to inhibit paclitaxel-induced apoptosis. Twenty-four hours following paclitaxel treatment, 697 pre-B cells and 697 cells overexpressing bcl-2 (697/Bcl-2) showed equivalent G2/M block and microtubule bundles resistant to calcium depolymerization. However, DNA fragmentation was observed in 697, but not 697/Bcl-2 cells at this time (24 h). But when cell were transferred to drug-free media, both 697 and 697/Bcl-2 cells lost viability (trypan blue) and became apoptotic, albeit with different time courses. (697: 50% viability at 48 h, 60% apoptotic at 72 h vs. 697/Bcl-2: 98% viability at 48 h, 50% viability at 160 h, 50% apoptotic at 216 h). Thus increased levels of bcl-2 delayed but did not prevent paclitaxel-induced apoptosis [32].

The role of other members of the Bcl-2 family has also been examined. Alternate splicing of the *Bcl-x* gene transcript produces bcl-x_L, an inhibitor of apoptosis, or bcl-x_S, a dominant negative inhibitor of bcl-2 and bcl-x_L. Transfection of MCF-7 cells with bcl-x_S increased paclitaxel sensitivity. Stable transfection had little effect on viability but increased DNA fragmentation in response to paclitaxel as well as to VP-16 treatment. Transient overexpression increased basal apoptosis and enhanced the DNA fragmentation observed in response to paclitaxel [33].

Finally, the role of the serine/threonine kinase Raf-1 in paclitaxel-induced bcl-2 inactivation and initiation of apoptosis has been pursued. Exposure to paclitaxel led to hyperphosphorylation of Raf-1 in 7 different cell lines and to bcl-2 phosphorylation in those cells with detectable bcl-2. Depletion of Raf-1 by geldanamycin in MCF-7 cells prevented paclitaxel-induced bcl-2 phosphorylation and also prevented paclitaxel-induced apoptosis measured by nuclear morphology after 16 h exposure. PMA exposure also induced Raf-1 phosphorylation but did not result in bcl-2 phosphorylation, occurred quickly as compared to the delayed response to paclitaxel, and did not prevent subsequent paclitaxel-induced bcl-2 phosphorylation. The results suggest that a distinct subcellular component of Raf-1 mediates

an apoptotic pathway involving bcl-2 and that this pathway is stimulated by paclitaxel [34].

2.5. *Resistance to taxanes*

Taxane resistance has been shown previously to be mediated by P-glycoprotein, and current efforts are directed at clarifying the mechanisms responsible for resistance in the absence of P-glycoprotein. Recent studies have focused attention on the potential role of altered isotype expression, albeit with conflicting results.

In MES-SA sarcoma cells exposed to 10 nM paclitaxel for 7 days, fluctuation analysis indicated a mutation to resistance rate of $5–8 \times 10^{-7}$/cell generation and a spontaneous mutation and drug selection mechanism for occurrence. Increased *mdr1* expression was found in 44% of the clones, without drug accumulation changes in the clones without *mdr1*, excluding other transport mechanisms. While tubulin levels were unchanged in seven of the nine resistant clones, total tubulin levels as measured by immunoblot with a pan-specific monoclonal antibody were reduced (50%) in one clone without *mdr1*, and increased (230%) in one with *mdr1*. All resistant clones, regardless of *mdr1* expression displayed 2- to 3-fold decrease in expression of the $\beta 4$ and 5β isotypes of β-tubulin, but no changes in α-tubulin isotype expression. All of the *mdr1* expressing clones but none of clones without *mdr1* were cross resistant to VP-16 and doxorubicin; but all nine clones were resistant to vinca alkaloids [35].

It can be argued that the consistent changes in isotype expression independent of resistance mechanisms in the MES-SA clones suggest these alterations resulted from paclitaxel selection, but were not directly involved in the resistance to paclitaxel. However, other studies suggest that isotype expression may be important. Using murine macrophage J774.2 cells, four cell lines were isolated (three with paclitaxel and one with docetaxel) and characterized. Increased *mdr1* expression was demonstrated in the four cell lines, with cross resistance to the other taxane, vinblastine, colchicine and doxorubicin. The most resistant line, J7-T1, was 2000-fold resistant and paclitaxel dependent. Total tubulin was increased in all lines about 2-fold based on immunoblots with monoclonal α- or β-specific antibodies. Isotypes M2, M3, and M5 were detectable by RT-PCR in parental and resistant cells. Expression of the M2 isotype was low in parental cells but was increased about 20-fold in the J7-T1 line, leading the authors to conclude that changes in isotype abundance can contribute to the development of taxane resistance [36].

Similarly, KPTA5, a paclitaxel resistant cell line derived from human leukemia K562 cells was 10-fold resistant to paclitaxel and docetaxel, minimally resistant to other agents, and showed similar morphology, doubling times, tubulin content, and paclitaxel accumulation as parental cells. Tubulin isotype expression was similar in parental and resistant cells with the exception of the minor 5β-isotype which was increased about 2-fold in the resistant cells at mRNA and protein levels, again leading the authors to conclude that alterations in expression of isotypes may contribute to paclitaxel resistance [37].

It is anticipated that future studies will clarify the role of isotype expression in resistance, while examining additional mechanisms including acquired tubulin mutations and alterations in the pathways leading to apoptosis.

2.6. *Combination with other agents and with radiotherapy: in-vitro and pre-clinical studies*

With the anticipated increase in clinical trials using combination regimens, in-vitro and preclinical models have been examined to provide

a basis for such efforts. The effects of combining paclitaxel or docetaxel with other agents has been investigated in a number of experimental models, and with different schedules. Combination with alkylating agents, cisplatin, doxorubicin, 5-fluorouracil (5-FU), and radiation have been reported. An additive response has been most often found when paclitaxel is administered first. Antagonism has been found in several combinations when paclitaxel is second. Potentiation of paclitaxel has also been found in combination with agents that independently affect microtubules: cyclocreatine and colchicine analogs.

Sequence dependent effects were observed with human gastric and ovarian carcinoma cell lines treated with paclitaxel in combination and in sequence with cisplatin using 2-h drug exposures. If paclitaxel treatment preceded cisplatin by 24 h, the treatments were additive. Simultaneous exposure, or treatment with cisplatin up to 72 h preceding paclitaxel resulted in antagonism of effects [38]. In contrast, in human melanoma G361 cells using 1 h exposure to cisplatin preceding or following 1 h or 24 h exposure to paclitaxel antagonism was found with all sequences, but was more pronounced with paclitaxel treatment first [39].

In an animal model, sequence effects were found in experiments with a transplantable ovarian carcinoma in mice. Drug effects were measured by regrowth delay following treatment with cisplatin and paclitaxel. Best results were obtained if paclitaxel preceded cisplatin i.p. Additive or greater than additive results were obtained with a treatment interval of 48 h. In the reverse sequence, additive or better results were obtained with a 48 h interval again found optimal, but greater morbidity and mortality resulted with this treatment sequence [40].

Combination of paclitaxel with doxorubicin also revealed sequence dependent effects in experiments with breast MCF7, colon WiDr, non-small cell lung cancer A549, and ovarian PA1 cells. Simultaneous or sequential exposure for 24 h to these agents was tested. Additive growth inhibition was found with all combinations in PA1 cells or with paclitaxel exposure first in the other cell lines. In the latter, simultaneous treatment, or paclitaxel after doxorubicin resulted in antagonism [41].

Paclitaxel with 5-FU in combination and in sequence has also revealed schedule-dependent interactions. Four cell lines were used: non-small cell lung cancer A549, breast cancer MCF7, ovarian cancer PA1, and colon cancer WiDr. These cells were exposed for 24 h to various concentrations of paclitaxel (0–10 nM) or 5-FU (0–20 μM), separately, simultaneously, or sequentially (24 h each). In addition, cells were continuously exposed to combinations for the 5 days. Sequential exposure to paclitaxel and then 5-FU yielded additive toxicity in all cell lines, while simultaneous (24 h) exposure or exposure to 5-FU followed by paclitaxel was mostly antagonistic, although results varied somewhat between cell lines. Long-term (5-day) continuous exposures were additive. These results indicate that combinations of paclitaxel and 5-FU are optimized by the sequence paclitaxel followed by 5-FU, but that prolonged simultaneous exposure may circumvent antagonism occurring with short term exposures [42].

Combination of paclitaxel with gallium nitrate provides an exception to the general observation that optimal results are obtained when paclitaxel precedes a second agent in a combination schedule. In the human breast carcinoma line, MDA-MB-435, paclitaxel and gallium nitrate toxicity was additive if exposure was simultaneous or if gallium was added after an initial paclitaxel-only exposure of up to 24 h or if gallium alone was added first for less than 16 h. If gallium alone for 16 or 24 h was followed by paclitaxel, greater than additive results were obtained in both assays [43].

Potentiation of effects has also been found in combination with other agents that affect microtubules. Cyclocreatine, which increases microtubule stability, is toxic to cells expressing high levels of creatine kinase. In a clonogenic assay with MCF-7 cells, 24 h treatment with minimally toxic 0.5 mM cyclocreatine in combination with a 1 h exposure at hour 5 to varying paclitaxel was compared to paclitaxel alone. Greater than additive toxicity was observed which increased at high paclitaxel concentrations [44].

Enhanced toxicity may also be obtained by combining a microtubule destabilizing drug with paclitaxel or docetaxel. Combination of the colchicine analog, colcemid, or the deazapteridine, CI 980, with docetaxel or paclitaxel altered microtubule polymerization in vitro by inducing production of short microtubules. Abnormal asters and short bundles were obtained with docetaxel/CI 980 combinations, with minimal concentrations of 5 and 1 nM in KB 3-1 cells. Enhanced G2/M accumulation was found by DNA content. Lower docetaxel concentrations resulted in multinucleated cells. Combination of 1 nM docetaxel with 0.05–0.25 nM CI 980 or 2.5–10 nM colcemid caused no change in polymerized tubulin content, but resulted in greater than additive cytotoxicity against KB 3-1 cells after 48 or 72 h [45].

Enhanced activity was also reported with combinations of paclitaxel and vinorelbine tested in a mouse model against P388 murine leukemia cells. Optimal combinations resulted in more than 80% 60-day cures (no tumor at 60 days), compared to $<3\%$ with either agent alone. The LD_{10} for vinorelbine was increased 2.5-fold by paclitaxel, allowing safe administration of otherwise toxic doses. Survival distribution was not affected by the sequence of the agents, when one hour separated them. The time between agents was significant. Dose combinations yielding 80% 60-day cures with 1 h separation were highly toxic when the interval was 6 or 24 h [46].

Finally, combination of paclitaxel and radiation has also been shown to result in increased effectiveness that is sensitive to schedule and may enhance radiosensitivity by increasing oxygenation due to tumor cell loss following paclitaxel-induced cell death [23]. Although there is some dispute as to how potent a radiosensitizer paclitaxel is [47], encouraging results in patients with unresectable non-small cell lung cancer are likely to maintain this issue under active investigation [48].

3. Clinical research

3.1. *Clinical studies*

Clinical development of paclitaxel has moved to up-front, and combination regimens, even as single agent activity continues to be explored. A role for paclitaxel in the treatment of ovarian cancer has been firmly established, with current efforts directed at resolving issues discussed in Section 3.2, as well as the best combination and route of administration. It can be expected that resolution of these issues should take time, but will not likely affect the activity of current 'best combinations' in a major way.

In ovarian cancer, GOG 111 showed the combination of paclitaxel (135 mg/m^2/24 h) and cisplatin (75 mg/m^2) to be superior to cyclophosphamide (750 mg/m^2) and cisplatin (75 mg/m^2) [49]. In women with suboptimally resected stage III and stage IV ovarian cancer, the paclitaxel containing regimen improved the duration of progression free survival and overall survival; even though the frequency of surgically verified compete responses was similar in the two groups. This is consistent with the emergence of microscopic resistant disease in the cyclophosphamide containing regimen,

and emphasizes that when two regimens are compared, even though similar complete response rates may be observed, differences in relapse and survival may appear. The latter would be expected if a combination of non-cross resistant drugs (paclitaxel and cisplatin) is compared with an equally efficacious combination of mechanistically similar agents (cyclophosphamide and cisplatin). This study confirmed what was becoming standard practice, but as the authors cautioned, "we are concerned that variants of the cisplatin-paclitaxel regimen are being adopted without proper evaluation".

A few studies begun at an earlier time that recruited paclitaxel naive patients further confirmed the activity of paclitaxel or paclitaxel containing regimens in ovarian cancer. The combination of cyclophosphamide (1250 mg/m^2) with paclitaxel (170–250 mg/m^2) was shown to be active in patients with recurrent adenocarcinoma of the ovary, with a response rate of 55% [50]. Although the authors cautioned that data supporting a superiority of combinations over single agent paclitaxel are lacking, they concluded that their findings were consistent with a clinically synergistic interaction between these two agents; a possibility that must be confirmed in a prospective trial. Similarly, activity of cisplatin (50 mg/m^2) in combination with paclitaxel (135 mg/m^2) in both platinum sensitive and refractory patients was reported, although again, the contribution of cisplatin must be confirmed [51]. However, the incorporation of paclitaxel into first line regimens means that future relapse populations will have been exposed to paclitaxel. In this regard, paclitaxel was shown to be active in the salvage setting in patients previously treated with paclitaxel [51,52]. Further studies will be needed to better define this activity, but the preliminary evidence suggests that the response to the previous regimen, the duration since prior exposure, and the doses administered will likely be important; an observation similar to that with other agents in ovarian and other cancers.

While the combination of cisplatin with paclitaxel is likely to be widely adopted, the search for less toxic regimens continues. Recognition that carboplatin, an analog of cisplatin with the potential advantage of reduced non-hematologic toxicity, is likely to be substituted for cisplatin in the community, has prompted the evaluation of combinations with paclitaxel. In a phase I study, it was demonstrated that carboplatin (AUC 7.5 or 470 mg/m^2) could be safely administered with paclitaxel (175 mg/m^2/3 h), or 135 mg/m^2/24 h), without G-CSF [53]. Ongoing trials seek to compare the activity of this regimen with that of other established therapies. Similarly, the desire to improve the activity of current regimens has been a catalyst for the exploration of more intense therapies to administer to poor prognosis ovarian carcinoma patients. Preliminary results indicate that the combination of cisplatin ($\geq$75 mg/m^2) cyclophosphamide (750 mg/m^2) and paclitaxel (250 mg/m^2) can be administered with G-CSF, albeit with measurable toxicity, to patients with bulky residual tumor [54]. The pathologic response rate of 36% with an additional 25% of patients having minimal microscopic disease is encouraging but must be confirmed in a prospective, randomized trial. While greater dose intensity may result in a higher initial response rate, the addition of cyclophosphamide, which does not add a significant 'new activity' to platinum/paclitaxel combinations, may not translate to more durable responses.

In breast cancer, paclitaxel's activity has received increased scrutiny, as a large number of combinations have been explored. While patient populations, paclitaxel schedules and intensity of regimens have been too diverse to

permit direct comparisons, it appears that numerous combinations are active and can be administered safely. Paclitaxel's activity in metastatic breast cancer, even in patients with prior anthracycline therapy, was confirmed in a randomized comparative study of two doses of paclitaxel [55]. As pointed out in that study: "the level of activity of single-agent paclitaxel is similar to the level of activity reported for the most commonly used drugs in the treatment of metastatic breast cancer following prior chemotherapy". In combination regimens, paclitaxel was administered with numerous agents previously shown to be active in metastatic breast cancer. In previously treated patients, these included: mitoxantrone, fluorouracil and high dose leucovorin [56]; cisplatin [57]; cyclophosphamide [58]; cyclophosphamide and cisplatin with autologous hematopoietic progenitor support [59]; epirubicin [60]; and 5-fluorouracil and folinic acid [61,62]. While a direct comparison of these regimens is not possible, their activity was similar (51% to 85%), and future studies will be needed to determine the combination with the highest activity and the least toxicity. The observation that prior anthracycline therapy was not an adverse factor suggests an anthracycline may be added to those regimens without an agent from this class of compounds. Should this be done, thought will need to be given to the design of the regimen to ensure optimum activity [63]

In addition to its documented activity in ovarian and breast cancer, paclitaxel has also shown activity in an impressive list of malignancies, all of which are currently undergoing evaluation: Non-small-cell lung cancer, where the role of adding carboplatin remains unresolved [64], and two Phase I trials with ifosfamide were encouraging [65,66]; urothelial carcinoma, where paclitaxel was shown to be effective in patients in whom prior cisplatin-based therapy failed [67]; adenocarcinoma of the endometrium [68]; malignant glioma [69]; and primary peritoneal carcinoma [70].

While experience with paclitaxel continues to exceed that with docetaxel, an increasing number of studies have appeared in which docetaxel has been found to have activity in a spectrum of malignancies similar to that in which paclitaxel has been previously found to be active. The latter includes: advanced breast cancer, in which the most experience is available, and in which activity has been shown in both refractory and previously untreated patients [71–73]; squamous cell carcinoma of the head and neck [74]; and non-small-cell lung cancer [75]. It is anticipated that an increasing number of studies using docetaxel will appear, and that with available information, the difference, if any, between these agents may become apparent, and their place in clinical oncology better defined.

3.2. Dosage and schedule issues

As we enter 1997, Arbuck's 1994 question: "Paclitaxel: What schedule? What dose?" remains unanswered.

The optimal schedule has yet to be defined. Both preclinical and clinical studies have demonstrated that prolonged paclitaxel exposure results in enhanced cytotoxicity, but the significance of this remains to be determined. Beyond efficacy, however, it is clear that convenience/ease of administration and toxicity are as important issues as activity. All things being equal, it is clear that a 3-h infusion would be preferable to the community at large. The resolution of acute hypersensitivity allergic reactions with premedication, independent of infusion duration, has eliminated this early problem from consideration. And while the toxicity profiles differ, increasing experience with paclitaxel has led to improved drug tolerance, so

that the toxicity issue may also become insignificant, at least in the majority of patients. Nevertheless, it is clear that while shorter paclitaxel infusions may cause less hematologic toxicity, the frequency and severity of some non-hematologic toxicities, especially neurotoxicity and myalgias, is higher.

Although the answer will be provided by randomized studies, preliminary studies indicate that at least in some patients, a 96-h infusion can effect a response in a tumor that previously did not respond to or progressed following a shorter infusion duration. Interestingly, for paclitaxel this was demonstrated in patients with breast cancer, where the drug has excellent activity, but not in malignant lymphoma, where its activity profile has been generally disappointing. This suggests that a change in schedule may only be able to improve efficacy in drug sensitive malignancies [76–78].

Similarly to schedule, the optimal dose has yet to be defined. While preclinical data can be cited that either supports or rejects the value of paclitaxel dose intensity, the desired randomized comparison has not yet been published. However, increasing evidence suggests that for both paclitaxel and docetaxel a dose response relationship exists, albeit modest and of uncertain clinical significance [65,66]. Given current data, we would not be surprised if future studies find higher initial response rates in patients receiving higher doses of taxanes, without a clinically significant prolongation of response duration or survival. The latter, we believe, will be achieved only with non-cross resistant combinations. These comparisons will be most meaningful if patients receiving lower doses are treated with what are now recognized as optimal doses. So that, for example, while in patients with metastatic breast cancer, 'superior efficacy' of higher doses has been reported, the lower dose of 135 mg/m^2 would generally not be regarded as optimal [55]; and the statistically significant increase in time to progression (3.8 vs. 2.9 months, $P = 0.009$) clinically insignificant, especially in the context of similar overall survival. This interesting study should not be extrapolated to mean that higher (and more toxic) doses will provide further benefit; rather it emphasizes the need to administer optimal doses avoiding where possible dose reductions.

For docetaxel, a dose–response relationship may also exist, although as with paclitaxel, convincing evidence is not yet available, nor its clinical importance understood. For example, while previous phase II trials of docetaxel in advanced non-small-cell lung cancer had reported response rates of 23% to 38% with a dose of 100 mg/m^2, and 25% at 75 mg/m^2, only 19% of patients treated at 60 mg/m^2 responded [75]. And in breast cancer, a response rate of 52% was obtained with a dose of 75 mg/m^2 compared with 68% in a previous trial with 100 mg/m^2 [72]. To be sure, the small size of these studies precludes definite conclusions from being reached, but suggest that further consideration to this variable should be given.

Finally, two studies reported more frequent paclitaxel administration as treatment alternatives. One interesting combination administered paclitaxel in combination with cisplatin, on a biweekly schedule. The starting dose of 90 mg/m^2, which was also the final dose, represents a dose intensity of 135 mg/m^2 every three weeks, and compares favorably with other regimens. The other administered weekly 1 h paclitaxel on six consecutive weeks, followed by one week of rest (50-day schedule). The MTD was reached at a dose level of 90 mg/m^2 comparable to 222 mg/m^2 every three weeks. While only mild toxicities were reported in heavily pre-treated breast and ovarian cancer patients, efficacy will have to be evaluated in a phase II study before comparisons with other regimens can be made [79].

3.3. *Pharmacokinetics*

Although the pharmacokinetics of the taxanes remain incompletely understood, investigations have focused on both the pharmacokinetics of the drugs, as well as their interactions with other agents. The abundance of in-vitro evidence suggests that maximum activity is observed when paclitaxel precedes other agents in a combination. Clinical studies have thus far been able to only speculate about the efficacy of combinations depending on the sequence of administration.

On a practical level, it was reported that calculation of exposures of paclitaxel based on blood paclitaxel may be complicated by partitioning of paclitaxel in the blood. Specifically, it was demonstrated that platelets afford a considerable sink for paclitaxel, with 40% of paclitaxel added to whole blood found concentrated in platelet. The 240-fold accumulation of paclitaxel over plasma was saturable, reached equilibrium in 15 min, and was dramatically reduced by cold (0°C). A binding constant of about 0.8 μM was obtained, prompting the authors to suggest that binding to platelet microtubules may contribute to inter-individual variability as well as dose-dependent variability in paclitaxel volumes of distribution [80].

Regarding pharmacokinetics, further insight was obtained to explain preliminary studies which had demonstrated a non-linear pharmacokinetics behavior in patients receiving paclitaxel. More than proportional increases in areas under the AUC and peak plasma levels suggested that both elimination and tissue distribution were saturable processes. While mathematical models describing this were developed, the reasons for this remained obscure. The demonstration of similar pharmacokinetics in mice provided a pre-clinical model to examine this [81]. These studies demonstrated that cremophor EL has a profound effect on the pharmacokinetics of paclitaxel in mice. Cremophor EL markedly reduced the clearance of paclitaxel, resulting in higher plasma levels, without affecting tissue levels. Measurements of cremophor EL levels in both the experimental mice and humans receiving a 3-h i.v. infusion of 175 mg/m^2 revealed similar cremophor EL levels, making it likely that as in mice, cremophor EL contributes substantially to the non-linear pharmacokinetics behavior in humans.

Pharmacokinetics interactions with other drugs also continue to be evaluated. With R-verapamil, similar observations to those made with cremophor EL were made: a delay in mean paclitaxel clearance and an increase in mean paclitaxel concentrations [58]. With doxorubicin, the sequence of a 24-h paclitaxel infusion relative to a 48-h doxorubicin infusion has been evaluated. When paclitaxel preceded doxorubicin, clearance of the latter was reduced by nearly one-third, with resultant stomatitis. In noting the interaction, the authors recommended that when paclitaxel (by 24-h infusion) and doxorubicin are administered sequentially, the latter should be given first. However, the anti-tumor efficacy of this sequence must be considered before it is adopted. As discussed in the section describing combination with other agents, this combination may not be optimal or even antagonistic. The latter is underscored by the authors in their discussion, which cites ongoing work demonstrating enhanced cytotoxicity when paclitaxel precedes doxorubicin in in-vitro studies with breast cancer cell lines. While this is noted in the context of host toxicity which 'may be increased synergistically', the anti-tumor effect must not be overlooked [82]. And with carboplatin, although less thrombocytopenia was observed when carboplatin was administered following paclitaxel, a PK effect could not be demonstrated [83].

An early phase I study had demonstrate delayed clearance with increased hematologic toxicity of paclitaxel when it was administered after cisplatin. Although the mechanism for this effect was unclear, the authors speculated it was most likely a result of altered hepatic or tissue metabolism, rather than a change in renal clearance. Lack of a significant renal effect is supported by studies in patients with urothelial cancer and a case report of paclitaxel administered to a patient on long-term hemodialysis, which demonstrated that paclitaxel could be administered to a patient receiving hemodialysis in the same dose schedule as in patients with normal renal function [67,84]

The role of cremophor in enhancing paclitaxel efficacy remains unresolved. A significant contribution to clinical efficacy based on its ability to block P-glycoprotein has been considered unlikely for the majority of malignancies in which activity has been demonstrated clinically. However, the demonstration that paclitaxel infusions of 3 and 6 h but not 24 h can result in sustained plasma cremophor concentrations that can effect reversal of P-glycoprotein mediated resistance in vitro may lead to a re-evaluation of its potential efficacy [85]. A final answer may require determination of tissue levels in humans, which if similar to mice may be below the limit of detection of even sensitive assays [81].

Finally, for docetaxel caution should be exercised in patients with liver disease or extensive liver involvement, in the same way that has been previously advised for paclitaxel [86]; and the demonstration that cytochrome *P*-450 isoenzymes of the CYP3A subfamily are involved in docetaxel biotransformation has clinical implications that should be taken into account in the design of combination chemotherapy regimens [87,88]

3.4. Clinical toxicities

Increasing experience with paclitaxel has rendered its administration safe and its side effects manageable. While the 24 h infusion schedule was originally adopted to reduce the risk of severe hypersensitivity reactions, it has become apparent that the major contributor to its lessened toxicity was the prophylactic medication schedules instituted simultaneously. Consequently, it is now felt that hypersensitivity reactions can be abrogated by appropriate prophylaxis, independent of the infusion duration.

Myelotoxicity, mucositis and neurotoxicity are recognized as side effects. Although neurotoxicity has been previously described, additional studies detailing the spectrum and frequency of this complication were reported. In women with advanced breast cancer, a phase II trial of paclitaxel 200 mg/m^2 administered as a 24-h infusion followed by cisplatin 75 mg/m^2 reported limiting neurotoxicity. Neurotoxicity was cumulative with a higher percentage of patients experiencing neurotoxicity as the number of cycles administered increased. The authors concluded that the higher incidence of neurotoxicity may have resulted from the higher doses administered, a conclusion supported by data from other studies [89]. While the higher incidence in this study may have been a result of the higher doses administered, a study in patients with gynecologic cancer using a 3-h paclitaxel infusion appears to reflect the previously observed higher incidence of toxicity with the 3-h infusion regimens [90]. In discussing the outcome of their trial, the authors noted that while this regimen was highly effective, more than "70% of treated individuals experienced some degree of neurotoxicity, including 20% who developed severe symptoms". A comparison of the incidence in this study to that observed in the GOG trials of cisplatin (75 mg/m^2) plus paclitaxel (135 mg/m^2) demonstrated a

much higher incidence of neurotoxicity, which the authors point out "was (likely) the direct result of high peak levels of paclitaxel achieved in the systemic compartment during and following the 3 h infusion at essentially the same time a second neurotoxin (i.e. cisplatin) was administered". This is likely to be correct, given the previous clinical experience which has shown a higher incidence of neurotoxicity in 3-h infusion regimens, with a close correlation with the concentration of paclitaxel achieved systemically. And the latter may be greater in the presence of cisplatin, which could lower the threshold concentration. Several options exist for managing this complication including the use of carboplatin, temporal spacing of the drugs, longer infusion times for paclitaxel, or the use of different paclitaxel schedules such as detailed in the schedule and dose section; but the results underscore further the caution of GOG investigators regrading the proven efficacy (with acceptable toxicity) of the regimen utilized in GOG 111 for ovarian cancer patients. Indeed, support for the advantage of a 24-h infusion regimen in combination with cisplatin can be found in an interesting phase I trial of high dose paclitaxel, cyclophosphamide and cisplatin with autologous hematopoietic progenitor cell support, in which the MTD of a 24-h paclitaxel infusion was determined to be 775 mg/m^2! [59]. While this dose exceeded the expectation of many, it represents and infusion rate of less than 97 mg/m^2/3 h.

In addition, for paclitaxel, several studies and case reports noted less frequently observed toxicities including: severe lymphocytopenia and interstitial pneumonia in patients with non small cell lung cancer treated with paclitaxel and simultaneous radiotherapy [91]; a 'recall' reaction at a site of previous paclitaxel administration [92]; paclitaxel induced radiation recall dermatitis [93]; severe neuropathy in patients with previous radiotherapy to the head and neck region [94]; and transient encephalopathy reported in patients receiving 175 mg/m^2 over 3 h [95] and in two patients in the above-mentioned trial who received 825 mg/m^2 over 24 h [59].

Finally, for docetaxel, while the experience is less extensive, increasing evidence suggests that doses of 60–75 mg/m^2 may be optimal, and that 100 mg/m^2 is too toxic [73,74,96,97]. Fluid retention remains a significant problem. And as with paclitaxel, neurotoxicity is frequently encountered [98].

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